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(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets

(11) Publication number:

**0 070 687**  
**A2**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 82303701.5

(51) Int. Cl.<sup>3</sup>: **G 01 N 33/58**

(22) Date of filing: 14.07.82

(30) Priority: 17.07.81 US 284399  
17.07.81 US 284401

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(43) Date of publication of application: 26.01.83  
Bulletin 83/4

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(84) Designated Contracting States: BE CH DE FR GB IT LI NL

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(54) Light-emitting polynucleotide hybridization diagnostic method.

(57) A hybridization diagnostic method is disclosed which uses light-labeled, single-stranded polynucleotide reagents for hybridizing with immobilized sample single-stranded polynucleotides. Light emission is related to the amount of target polynucleotide in the sample.

**EP 0 070 687 A2**

LIGHT-EMITTING POLYNUCLEOTIDE  
HYBRIDIZATION DIAGNOSTIC METHOD

BACKGROUND OF THE INVENTION

5           Presently, nucleic acid hybridization assays  
are primarily used as a research tool for the detec-  
tion and identification of a unique deoxyribonucleic  
acid (DNA) sequence or a specific gene in a complete  
DNA, a mixture of DNA's, or a mixture of DNA frag-  
10           ments. A number of variations of the technique  
exist as used in recombinant DNA research (Methods  
in Enzymology, Vol. 68, R. Wu (Ed.) pp 379-469,  
1979; and Dunn, A. R., and Sambrook, J., Methods in  
Enzymology, Vol. 65, Part 1, 468-478, 1980). One  
15           of the most widely used procedures is called the  
Southern blot filter hybridization method (Southern,  
E., J. Mol. Biol. 98, 503, 1975). This procedure  
is usually used to identify a particular DNA frag-  
ment separated from a mixture of DNA fragments by  
20           electrophoresis. The procedure is generally carried  
out by subjecting a sample of DNA isolated from  
some organism to restriction endonuclease digestion  
and then electrophoresing the sample on a gel  
(agarose, acrylamide, etc.). The gel containing  
25           the separated DNA fragments is placed in contact  
(blotted) with a nitrocellulose filter sheet (or  
diazotized paper, etc.). The DNA fragments are  
transferred and become bound to the nitrocellulose  
sheet. The gel-transfer nitrocellulose sheet con-  
30           taining the DNA fragments is then heated (95°C) to  
denature the DNA. At this point the sheet is treated  
with a solution containing a denatured radio labeled  
(<sup>32</sup>p) "specific DNA probe" and hybridization is  
allowed to take place. Hybridization can take from  
35           3-48 hours, depending on given conditions, and un-  
hybridized "specific DNA probe" is washed away.  
The nitrocellulose sheet is then placed on a sheet

of X-ray film and allowed to expose, usually for several days at  $-70^{\circ}\text{C}$ . The X-ray film is then developed. The exposed areas on the film identify which DNA fragments have hybridized and thus have a sequence similar to that of the "specific DNA probe."

This procedure, as well as most other variations, requires the use of radioisotopes and is obviously very complex and time consuming. Because of these and other problems, DNA hybridization assays have remained only as a tool for basic research and have not been generally used in applied or commercial areas, such as for clinical diagnostics.

It would be very desirable to have a hybridization method which would avoid some of these problems, particularly the use of radioisotopes. Hybridization assays could be a valuable diagnostic method for identification of pathogenic organisms and for the determination of antibiotic resistant traits (genes) in microorganisms. They could also be used for genetically based human cell typing systems and for diagnosis of genetic diseases.

#### SUMMARY OF THE INVENTION

In general, the invention relates to a hybridization assay which is based on the inherent high fidelity of the base recognition process in complementary double-stranded (ds) nucleic acids (DNA, RNA, DNA-RNA, and synthetic polynucleotides). It involves a hybridization system that does not use radioisotopes, but instead uses chemiluminescent, fluorescent, or phosphorescent moieties which under proper conditions can provide sensitivity equal to that of radioisotopes but without the inherent problems. This invention has advantages over present methods which are too complex and time consuming to be used practically in clinical diagnostics.

In one aspect, the invention resides in a diagnostic method for determining the presence of and

the identification of viruses, bacteria, and other microorganisms, as well as the existence of certain genetic traits by assaying for a target nucleotide sequence which is uniquely characteristic of the target microorganism or genetic trait being assayed.

5 In particular, the method comprises the steps of (a) immobilizing the sample single-stranded polynucleotides on a suitable support; (b) contacting the immobilized sample, under hybridization conditions, with light-labeled reagent single-stranded polynucleotide segments which are complementary to a representative portion of the target single-stranded polynucleotide; (c) separating unhybridized reagent single-stranded polynucleotide segments from the immobilized sample; (d) exposing the immobilized sample to means for exciting the light label; and (e) detecting the light response from the immobilized sample.

20 The methods of this invention are directed towards the single-stranded polynucleotides because of their ability to combine with complementary strands in a highly specific manner. The single-stranded polynucleotides useful for purposes of this invention are readily obtained from double-stranded polynucleotides by denaturing, i.e. splitting the double-stranded polynucleotide into two complementary strands. This is typically accomplished by heating to about 70-100°C for about 5-10 minutes. The "target" single-stranded polynucleotide mentioned above is either of the two single-stranded polynucleotides obtained by denaturing that double-stranded polynucleotide (the target double-stranded polynucleotide) at which the diagnosis is ultimately directed, such as a particular gene. Similarly, the "single-stranded polynucleotide sample" can be obtained by isolating the double-stranded polynucleotides from the physiological sample to be diagnosed (blood,

urine, other body fluids, etc.) and denaturing the double-stranded polynucleotides to form the corresponding single strands.

5 Immobilization of the sample single-stranded polynucleotides can be accomplished by any suitable method which does not inactivate a significant number of the bases in the polynucleotide sequence, since a representative intact sequence must be available for base pairing with the reagent strands. The  
10 single-stranded polynucleotide segment can be attached or immobilized to a variety of supports, some of which include activated glass beads, polyacrylamide, agarose or sephadex beads, and cellulose. Numerous methods exist for coupling either terminal end of a  
15 polynucleotide to a given support (Weissback, A., and Poonian, M., Methods in Enzymology, Vol. XXXIV, Part B, 463-475, 1974). Also, derivatized forms of polynucleotides, for example, one carrying a terminal aminohexyl nucleotide, can be easily attached on a  
20 variety of supports (Mosbach, K., et al., Methods in Enzymology, Vol. XLIV, 859-886, 1976). Oligoribonucleotides may be immobilized on boronate derivatives of various supports (Schott, H., et al., Biochemistry, 12, 932, 1973).

25 Hybridization conditions necessary to accomplish base pairing of the target single-stranded polynucleotide in the sample (if present) and the reagent single-stranded polynucleotide segments are determined by a variety of factors, such as the nature of the  
30 light label attached to the reagent polynucleotide sequences, the size of the reagent polynucleotide sequences, the [G] + [C] (guanine plus cytosine) content of the reagent and sample polynucleotide sequences, and how the sample polynucleotide sequence  
35 is prepared. The light label can affect the temperature and salt concentration for the hybridization reaction. Chemiluminescent catalysts can be sensi-

tive to temperatures and salt concentrations that absorber/emitter moieties can tolerate. Size of the reagent polynucleotide sequences affects the temperature and time for the hybridization reaction.

5 Assuming similar salt and reagent concentrations, hybridization involving reagent polynucleotide sequences in the range of 10,000 to 100,000 nucleotides might require from 40 to 80 minutes to occur at 67°C, while hybridization involving 20 to 100 nucleotides would require from 50 to 30 minutes at 25°C. 10 Similarly, the [G] + [C] content of the reagent and sample polynucleotide sequences affects the temperature and time for the hybridization reaction. Polynucleotide sequences with a high [G] + [C] content 15 will hybridize at lower temperatures in a shorter period of time than polynucleotide sequences with a low [G] + [C] content. Finally, conditions used to prepare the sample polynucleotide sequence and maintain it in the single-stranded form can affect the 20 temperature, time, and salt concentration of the hybridization reaction. The conditions for preparing the sample polynucleotide sequence are affected by polynucleotide length required and the [G] + [C] content. In general, the longer the sequence or 25 the higher the [G] + [C] content, the higher the temperature and/or salt concentration required.

Reagent single-stranded polynucleotide segments must be complementary to a representative portion of the target single-stranded polynucleotide so as 30 not to give a false indication of a positive test in the absence of the target single-stranded polynucleotide in the sample. The term "representative" is intended to be an imprecise term since the desired accuracy of the method will vary with the purpose. 35 Ideally, the reagent single-stranded polynucleotide will only pair with target single-stranded polynucleotide and will do so whenever the latter is present

in the sample. If the target single-stranded polynucleotide base sequence is very unique, very high accuracy can be obtained. Preferably, the reagent single-stranded polynucleotide segments are prepared from the target double-stranded polynucleotide, which is denatured and separated into the two complementary single strands. Either single strand can be chosen as the reagent material to be light-labeled, but only one of them can actually be used in order to prevent the reagent from hybridizing with itself. The sample single-stranded polynucleotides need not be separated in this manner, however, in which case only half of the single-stranded polynucleotides present in the sample, which were derived from the target double-stranded polynucleotide, would be suitable hybridization candidates. If sequences are known, then reagent single-stranded polynucleotides can be made by organic synthetic techniques.

Light labels can be chemiluminescent, bioluminescent, phosphorescent, or fluorescent and can be attached to any point on the reagent single-stranded polynucleotide segments; however, terminal positions are more desirable. Suitable light labels of the chemiluminescent type include, without limitation, peroxidase and iron porphyrin derivatives. Suitable light labels of the bioluminescent type include, without limitation, bacterial luciferase (either one or both luciferase and associated dehydrogenase), firefly luciferase, flavin mononucleotide (FMN), adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and various long chain aldehydes (decyl aldehyde, etc.). Suitable fluorescent light labels include, without limitation, a variety of fluorescent nucleotides (etheno-adenosine nucleotides, etheno-cytidine nucleotides,



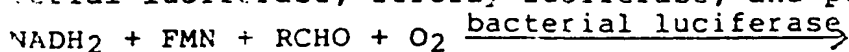
etc.) or functionalized nucleotides (amino-hexane adenosine nucleotides, mercurinucleotides, etc.) which could first be fluorescently labeled and then covalently attached to the reagent single-stranded polynucleotide segment. A variety of methods exist for preparing fluorescent derivatives of oligoribonucleotides (Annual Review Biophys. Bioeng., A. Faure and G. Thomas, pp. 175-195, 1981). Suitable phosphorescent light labels include, without limitation, 2-diketones such as 2,3-butadione, 1-[carboxyphenyl]-1,2-pentanedione, and 1-phenyl-1,2-propanedione. Means for attaching the light labels to the reagent single-stranded polynucleotide segments are documented in the art.

In order to determine if hybridization has occurred and hence the presence of the target polynucleotide in the sample, it is first necessary to remove the unhybridized light-labeled reagent. This is most simply accomplished by washing or flushing with appropriate buffer solution. Complete removal of unhybridized reagent is a goal to be attained, but is not likely since there will be some residual reagent which sticks to the immobilization support or possibly partially hybridizes with the sample. These occurrences will cause "background" when measuring the light response and must be accounted for when analyzing the results of the test.

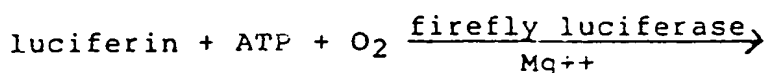
After the separating step, the immobilized sample must be exposed to a means for exciting the light labels, if present, which may have become immobilized through hybridization between the reagent and immobilized sample single-stranded polynucleotides. In the case of fluorescent or phosphorescent labels, the sample need only be irradiated with the appropriate light wavelength. This technique is well known in the art. In the case of the chemiluminescent or bioluminescent labels, the sample must be exposed

to a light reagent system which depends specifically on the light label which has been used. Such light reagents are also well known in the art. For example, the following reactions illustrate how a light re-

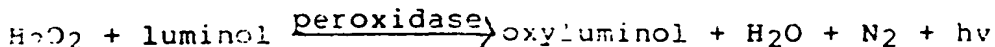
5 response is elicited using three different light-emitting catalysts as the light labels, namely bacterial luciferase, firefly luciferase, and peroxidase:



10



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In the presence of an excess of reactants, the light response will vary with the number of light-labeled reagent single-stranded polynucleotide segments which have become hybridized with the immobilized sample. The choice of the label will be determined by ease of labeling and the economics of the light reagent system.

25 The detection of the light response can be accomplished with numerous detection devices which are commercially available. For example, using photomultiplier detection devices, NADH<sub>2</sub> levels of 10<sup>-3</sup>-10<sup>-9</sup> mg./ml., ATP levels of 10<sup>-3</sup>-10<sup>-11</sup> mg./ml., and H<sub>2</sub>O<sub>2</sub> levels of 10<sup>-3</sup>-10<sup>-7</sup> mg./ml. can be measured.

30 As mentioned above, it is necessary to immobilize the sample single-stranded polynucleotide on a support. In addition to the aforementioned methods of immobilization, the sample single-stranded polynucleotide may be immobilized by contacting the sample under hybridization conditions with an immobilized  
35 single-stranded polynucleotide reagent segment, said first single-stranded polynucleotide reagent segment being complementary to a portion of the target single-stranded polynucleotide. When target

single-stranded polynucleotide is present in the sample it hybridized with the immobilized first single-stranded polynucleotide reagent segment. The target single-stranded polynucleotide then serves  
5 as a template for hybridization with the light-labeled second single-stranded polynucleotide reagent segment which is complementary to a substantially mutually exclusive portion of the target single-stranded polynucleotide relative to said first single-stranded polynucleotide reagent segment. This system  
10 has the advantage of improved specificity because it involves two hybridization processes.

The first and second single-stranded polynucleotide reagent segments must consist essentially of  
15 bases which are complementary to the base sequence of the target single-stranded polynucleotide. It is necessary that the first and second segments be complementary to substantially mutually exclusive portions of the target single-stranded polynucleotide.  
20 In other words, upon hybridization with the target polynucleotide the first and second reagent segments should not compete for the same base sequence to the extent that hybridization is prevented. In some cases, the first and second segments will line  
25 up head to tail (3' end to 5' end) with no overlap and with few or no basepairing spaces left between them. However, in other cases it will be highly desirable (because it would improve specificity) that the first and second single-stranded polynucleotide reagent segments be complementary to two widely  
30 separated but unique sequences on the target single-stranded polynucleotide.

First and second single-stranded polynucleotide reagent segments can be prepared from appropriate  
35 restriction endonuclease treatment of target nucleic acids from the organism of interest or, in cases where the base sequence of a unique portion is known,

they can also be synthesized by organic synthetic techniques (Stawinski, J., et al., Nuc. Acids Res. 4, 353, 1977; Gough, G. R. et al., Nuc. Acids Res. 6, 1557, 1979; Gough, G. R. et al., Nuc. Acids Res. 7, 1955, 1979; Narang, S. A., Methods in Enzymology, Vol. 65, Part I, 610-620, 1980).

The size of the reagent segments can be from 10 nucleotides to 100,000 nucleotides in length. Hybridized systems having fewer than 10 nucleotides are not stable and will begin to denature about 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, (See Molecular Genetics, G. S. Stent and R. Calendar, pp 213-219, 1971). Ideally the reagent segments should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) would lend themselves to production by automated organic synthetic techniques. Sequences from 100-100,000 nucleotides could be obtained from appropriate restriction endonuclease treatments. Ideally small sequences should be fluorescent labeled, while larger sequences could be chemiluminescent labeled. The labeling of the smaller segments with the relatively bulky chemiluminescent moieties may in some cases interfere with the hybridization process.

It will be appreciated by those skilled in the art that many variations from the foregoing can be made without departing from the scope of this invention.

We claim:

1. A method for assaying target single-stranded polynucleotides in a single-stranded polynucleotide sample comprising:

- 5 a) contacting an immobilized sample single-stranded polynucleotide on a support, under hybridization conditions, with light-labeled reagent single-stranded polynucleotide segments which are complementary to a representative portion of the target single-stranded polynucleotide;
- 10 b) separating unhybridized reagent single-stranded polynucleotide segments from the immobilized sample,
- c) exposing the immobilized sample to
- 15 means for exciting the light labeled; and
- d) detecting the light response from the sample.

2. The method of Claim 1 wherein (a) the sample single-stranded polynucleotides are immobilized by

20 contacting the sample, under hybridization conditions, with immobilized first single-stranded polynucleotide reagent segments being complementary to a portion of the target single-stranded polynucleotides, and

(b) the light-labeled reagent single-stranded polynucleotide segments are light-labeled second single-stranded polynucleotide reagent segments which are

25 complementary to a substantially mutually exclusive portion of the target single-stranded polynucleotide relative to said first single-stranded polynucleotide reagent segment.

30

3. The method of Claim 1 or Claim 2 wherein the light label is a chemiluminescent label.

4. The method of Claim 3 wherein the chemiluminescent label is selected from the group consisting

35 of peroxidase and iron porphyrin derivatives.

5. The method of Claim 1 or Claim 2 wherein the light label is a fluorescent label.

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6. The method of Claim 5 wherein the fluorescent label is selected from the group consisting of etheno-adenosine nucleotides, etheno-cytidine nucleotides, amino-hexane adenosine nucleotides, and mer-  
5 purinucleotides.

7. The method of Claim 1 or Claim 2 wherein the light label is a bioluminescent label.

8. The method of Claim 7 wherein the bioluminescent label is selected from the group consisting of  
10 bacterial luciferase, firefly luciferase, flavin mononucleotide, adenosine triphosphate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, and decyl aldehyde.  
15

9. The method of Claim 1 or Claim 2 wherein the light label is a phosphorescent label.

10. The method of Claim 9 wherein the phosphorescent label is selected from the group consisting of  
2,2-butadione, 1-[carboxyphenyl]-1,2-pentanedione,  
20 and 1-phenyl-1,2 propanedione.